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ARMY PROJECT ORDER NO: 91PP1807

TITLE: SUPPORT BETWEEN USAMRDC AND USDA FOR COOPERATIVE RESEARCH UNDER THE RATION SUSTAINMENT TESTING PROGRAM

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REPORT DATE: December 31, 1992

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

AGENCY USE ONLY | REPORT DATE | REPORT TYPE AND DATES COVERED

31 December 1992 Final Report (7/1/91 - 9/30/91)

TITLE AND SUBTITLE | FUNDING NUMBERS

Support Between USAMRDC and USDA for Cooperative Research Under the Ration Sustainment Testing Program

Army Project Order 91PP1807

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SUPPLEMENTARY NOTES

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We examined the effects of high energy expenditure and hypocaloric intakes during US Army Ranger training on cellular immune functions and systemic cytokine production. Blood samples were collected at baseline (B), and at the end of each of 4 phases, (Benning, I; Mountain, II; Jungle, III; Desert, IV), of training. Mitogen induced T-lymphocyte proliferation was suppressed at the end of Phases I-IV, with the greatest at the end of II and III. Mitogen induced interleukin-2 (IL-2) and soluble IL-2 receptor (IL-2R) levels were only decreased at the end of Phases I and II. Delayed type hypersensitivity (DTH) skin tests were conducted at B and at the end of phases III and IV. Ranger training did not impair the ability of the trainees to express DTH at the end of Phases III and IV to infectious microorganisms that they had immunity to at B. Whole-blood produced interleukin-6 (IL-6) in vitro was decreased in the trainees upon completion of Phase III, while plasma IL-6 concentration was increased at the end of Phase I, followed by continued reductions at the end of Phases of II-IV. Compared to B, Ranger training had a profoundly suppressive effect on T-lymphocyte function and systemic circulation of IL-6.

T-lymphocyte proliferation, Interleukin-2, Interleukin-2 receptors, delayed type hypersensitivity skin test, interleukin-6

SECURITY CLASSIFICATION OF REPORT	SECURITY CLASSIFICATION OF THIS PAGE	SECURITY CLASSIFICATION OF ABSTRACT	LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Tim L. Kramer 12/31/92

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INTRODUCTION

The US Army Ranger Training Course is a demanding training program which subjects trainees to multiple stressors including restricted food availability, sleep deprivation, and periods of sustained physical exertion. In early 1991, a Ranger class experienced a significant outbreak of pneumococcal pneumonia (1). Six percent (14 cases) of the trainees were diagnosed as clinical pneumonia and over 33% were identified as Streptococcus pneumoniae carriers, with 91% of the carriers reporting cough or rhinorrhea. These observations indicated that Ranger trainees exhibited a higher S. pneumoniae carrier rate than the general population. It was unclear if the higher rate resulted from greater environmental exposure, or impaired immunocompetence to infectious agents by the trainees. This pneumonia outbreak raised concerns that the multiple stressors associated with Ranger student training, specifically reduced food intake and accompanying body weight losses, might attribute to an unacceptably high susceptibility to illness from infectious agents.

While we know that protein/calorie malnutrition and single nutrient deficiencies impair in vivo and in vitro markers of immunocompetence (2-4), and increase the incidence of nosocomial infections in certain populations (5,6), no comprehensive data are available which describe these changes in soldiers undergoing Ranger training. Thus, it is not possible to determine if the reduced food intake and accompanying weight loss is of sufficient severity to increase the susceptibility of the Ranger trainee to infectious disease, and if so, what would constitute an appropriate nutritional or non-nutritional intervention to correct the condition.

The present contract was established to provide a longitudinal description of the nutritional and physiological impact c the Ranger course on in vitro and in vivo immune function of the trainees.

METHODS

Detailed methods used in this study, including immunological procedures, were described (7). Presented is description of the immunological procedures.

Cell-mediated immune functions

At each collection point, a blood sample was collected into siliconized vacutainer tubes containing sodium heparin (143 USP units) for immune studies. The blood was held at ambient temperature (18-27^{O}C) for 24 h prior to preparation for in vitro cell culture. This timing was designed to equate the handling of samples from each phase by accommodating the longest period of travel between test sites and the immu[illegible]gy laboratory in Beltsville, Maryland. Plasma for cytokine quantitation was removed from packed cells at approximately 32 h post-collection. The plasma samples were held at -70^{O}C until analysis.

Whole blood was diluted 1:4 and 1:2 with RPMI-1640 tissue culture medium (Sigma Chemical Co., St. Louis, MO) in polystyrene tubes for lymphocyte proliferation and cytokine production cultures, respectively. The RPMI-1640 contained L-glutamine at 2.0 mmol/L and penicillin-streptomycin at 100 U/mL and 100 ug/mL, respectively. The in vitro cultures for proliferative responsiveness received in order: 100 uL of RPMI-1640 per well of round bottom 96-well tissue culture plates, 50 uL of RPMI-1640 alone

(background) or with designated stimulant: phytohemagglutinin-M (PHA-M; 0.08 ug/uL, Sigma), conconavalin-A (ConA; 0.08 ug/uL, Sigma), pokeweed mitogen (PWM; 0.008 ug/uL, Sigma), or tetanus toxoid (TT; 0.128 Lf/uL, Connaught Laboratories International, Willowdale, Ontario, Canada), and 50 uL of blood (diluted 1:4). The cultures contained a final volume of 200 uL, with the final blood dilution at 1:16.

Proliferative activity in vitro was based on mean DNA incorporation of tritiated thymidine (methyl-^{3}H; specific activity 6.7 uCi, 248 GBeq/mmol, New England Nuclear, Boston, MA) by cells in triplicate cultures without (background) and with stimulant. PHA-M and ConA were added to the cultures at 4 ug, and PWM at 0.4 ug, per culture. TT was added to the cultures at 6.4 limits of flocculation (Lf) per culture. Cell cultures stimulated with PHA-M and ConA were incubated for 72 h, and those stimulated with PWM and TT for 144 h, at 37^{o}C in a 5% CO_2, 95% humidified air incubator. ^{3}H-thymidine (1.0 uCi; 37 KBeq) was added to each culture 24 h prior to termination, after which cells were harvested, the ^{3}H-thymidine labeled DNA was collected on 12-well filtermats (Skatron Inc., Sterling, VA), 4.5 mL of scintillation fluid (Ready-Safe, Beckman) was added to each vial containing individual filter discs, and the vials were counted (Beckman LS 3801 scintillation counter). Proliferation activity of lymphocytes is expressed as corrected dpm (stimulated minus background).

For cytokine production the cultures received the same treatment as the proliferative activity cultures, with PHA-M, ConA, or PWM stimulants but with 50 uL of blood diluted 1:2 instead of 1:4. The cultures also contained a final volume of 200 uL, but with the final blood dilution at 1:8. The cell cultures were incubated similarly to those for lymphocyte proliferation, except for 48 instead of 72 h for PHA-M and ConA-stimulated

cultures, and 96 instead of 144 h for PWM-stimulated cultures. The supernatants from each set of triplicate cultures were collected, pooled, and stored at -70°C until assayed.

Cytokine interleukin-2 (IL-2) was determined in supernatants from unstimulated and PHA-M-stimulated cultures by a commercially available ELISA (Dupont, Boston, MA). Cytokine interleukin-6 (IL-6) was determined in supernatants from unstimulated cultures and plasma by ELISA test kits (Clinigen, R&D Systems, Minneapolis, MN). Soluble IL-2 receptors (IL-2R) were determined in supernatants from PHA-M stimulated cultures using the CELL FREE Interleukin-2 Receptor Test Kit (T-Cell Sciences Inc., Cambridge, MA).

Delayed skin hypersensitivity test

Changes in the ability of soldiers to express an in vivo immune response was assessed by administering a delayed skin hypersensitivity test (Multitest-CMI, Connaught Laboratories, Inc., Swiftwater, PA) to each soldier during the baseline measurement period, and at the end of training phases III and IV. It contained a glycerin negative control and seven antigens of culture filtrate from the following microorganisms: Clostridium tetani (tetanus toxoid), Corynebacterium diphtheriae (diphtheria toxoid), Streptococcus, Group C (streptococcus), Mycobacterium tuberculosis (tuberculin, old), Candida albicans (candida), Trichophyton mentagrophytes (trichophyton), and Proteus mirabilis (proteus).

The tine tests were applied to the ventral forearm of each soldier in the morning, but after blood samples had been taken. After forty-eight hours, the response to each antigen was determined by measuring the diameters (parallel and perpendicular to the

long axis of the forearm) of the induration resulting at each of the eight tine administration sites. The site was recorded as a positive reaction when it showed an induration of 2 mm in diameter or more, compared to the negative control.

RESULTS AND DISCUSSION

Delayed skin hypersensitivity test

Delayed-type-hypersensitivity (DTH) skin-test reaction is an in vivo test for immune competence and function of T-lymphocytes and macrophages. A positive DTH response confirms previous exposure of the individual to the test antigen. The exposure can be environmental contamination and or prophylactic immunization. DTH skin-test responsiveness of the Ranger students at baseline and upon completion of the jungle and desert phases to antigens of 7 microorganisms is presented in Table 1. Compared to baseline, the percent number of trainees showing positive DTH skin-test responses to the test antigens tetanus, candida, streptococcus, and tricophyton did not change during the course. Increases were found in the percent number of trainees showing positive DTH skin-test responses to test antigens diphtheria, at the end of the jungle phase, proteus, at the end of both the jungle and desert phases, and tuberculin, at the end of the desert phase. These results demonstrate that T-lymphocyte function in the DTH skin-test reaction is maintained by trainees during the Ranger course. In summary, Ranger training does not impair at the end of jungle and desert phases the ability of the trainee to express cellular immunity to infectious microorganisms that they had immunity to prior to the start of training.

Table 1. Delayed-type-hypersensitivity skin test responsiveness at baseline and at the end of jungle and desert phases.

Antigen	Baseline	Jungle	Desert
Tetanus	91	87	85
Diphtheria	56[a]	74[b]	65[a,b]
Candida	56	56	56
Proteus	47[a]	65[b]	69[b]
Streptococcus	24	18	25
Tuberculin	16[a]	16[a]	31[b]
Tricophyton	15	24	15

Percent positive skin-test sites; induction $\geq$ 2 mm in diameter.
Values for each antigen marked with unsimilar letters are different ($p<0.05$).

Cellular Immune Functions In Vitro

T-lymphocytes are the immune specific cells involved in the DTH reaction. Since it is difficult to evaluate T-lymphocytes individually in the complex in vivo DTH reaction, in vitro tests have been developed to evaluate the function of these cells. T-lymphocytes cultured in vitro undergo proliferation when stimulated with certain lectins, such as pokeweed mitogen (PWM), phytohemagglutinin (PHA) or concanavalin-A (ConA), or antigens, such as tetanus toxoid (TT), to which the individual has been immunized.

Proliferative activity in vitro of lymphocytes from trainees at baseline, and upon completion of Benning, mountain, jungle and desert phases stimulated with PWM, PHA, ConA, or TT are shown in Figure 1. Compared to baseline, proliferative responsiveness in vitro of T- and B-lymphocytes to PWM was significantly decreased in the trainees upon completion of the Benning phase, followed by additional and equivalent reduction upon completion of the mountain and jungle phases. A partial recovery, equivalent to

Benning phase levels, was observed at the end of the desert phase. Similar results were observed for T-lymphocytes stimulated with PHA-M, which primarily stimulates T-lymphocytes. With minor exceptions, proliferative responsiveness of T-lymphocytes from the trainees to ConA (a T-lymphocyte mitogen) was similar to those for T-lymphocytes stimulated with PWM or PHA-M. Regardless of the mitogenic stimulant, T-lymphocyte proliferation was partially (PWM, PHA-M) or fully recovered (ConA) upon completion of the desert phase. Proliferation of T-lymphocytes to the antigen TT was suppressed in trainees at the end of the mountain phase, but recovered at the end of the jungle and desert phases.

The cytokine interleukin-2 (IL-2) is synthesized and secreted primarily by T-helper lymphocytes which have been stimulated with certain mitogens or antigens. Binding of IL-2 to receptors for IL-2 (IL-2R) induces clonal expansion of antigen-specific T-lymphocytes involved in immune protection against infectious diseases. Release of soluble IL-2R and the production of IL-2 by T-lymphocytes stimulated with PHA-M from the trainees at baseline, and upon completion of Benning, mountain, jungle and desert phases are presented in Figures 2a and 2b. Compared to baseline, IL-2R and IL-2 were significantly decreased by T-lymphocytes from trainees upon completion of the Benning and mountain phases. IL-2R and IL-2 were fully recovered to baseline level at the end of the jungle phase, and IL-2 was significantly increased above baseline upon completion of the desert phase. Similar to IL-2R and IL-2, proliferation of T-lymphocytes stimulated with PHA (Figure 2c) shows suppressed T-lymphocyte function by the Ranger trainees during the course. The suppressed T-lymphocyte functions in vitro appear to be primarily due to energy deficit. It also appears that early responses (IL-2R and IL-2) of T-lymphocyte activation/function normalize quicker to improved energy status than the late response, i.e., proliferation/DNA synthesis.

The cytokine interleukin-6 (IL-6) is a multifunctional protein produced by lymphoid and non-lymphoid cells. The primary sources of IL-6 from unstimulated whole-blood cultures would be T-helper lymphocytes and monocytes/macrophages. These cells plus fibroblasts, hepatocytes, and vascular endothelial cells would be the primary sources of IL-6 in plasma. Although IL-6 is a multifunctional cytokine it has a major role in the mediation of the inflammatory and immune responses initiated by infection or injury. Production of IL-6 by unstimulated whole-blood cultures from trainees at baseline, and upon completion of the Benning, mountain, jungle and desert phases are presented in Figure 3. Compared to baseline, whole-blood produced IL-6 was significantly decreased in the trainees upon completion of the jungle phase. Concentrations of IL-6 in cell culture supernatants were lower in trainees upon completion of the Benning, mountain and desert phases than at baseline, but not significantly.

Levels of IL-6 in plasma of trainees at baseline, and upon completion of the Benning, mountain, jungle and desert phases are shown in Figure 3. Compared to baseline, plasma IL-6 concentration was significantly increased at the end of the Benning phase, followed by significant decreases at the end of the jungle and desert phases. From the Benning through desert phase the trainees showed continued decreases in plasma IL-6 at each phase.

In summary, Ranger training had a suppressive effect on cellular functions of the immune protective system. The suppression was greatest at the time of highest energy expenditure and largest energy deficit (7). This preceded the periods of increased incidences of infections (jungle and desert phases) in the trainees (7).

CONCLUSIONS

A significant finding in this descriptive study is that aspects of in vitro immune function are suppressed in the second half of Ranger training. This includes decreased T-lymphocyte function and suppressed systemic production of interleukin-6. This is the likely reason for increased infection rates during the second half of the course. Extensive exposure to pathogens during immersion in the swamps in the jungle phase increases the opportunity for infection and makes this a highly predictable period of infection risk, especially with respect to cellulitis of the lower extremities. Prolonged energy deficit is a likely cause of the reduction in immunocompetence, probably working through other deficits which are likely to include a shortage of proteins important in immune function.

In summary:

Immunological responses were compromised, probably due to the reduced plane of nutrition. The decreased T-lymphocyte function and suppressed systemic production of interleukin-6 indicate that Ranger students have a decreased protection against infectious disease. It is not known if these immune deficits are continuous during each phase, or if they recover in each phase when energy intake increases.

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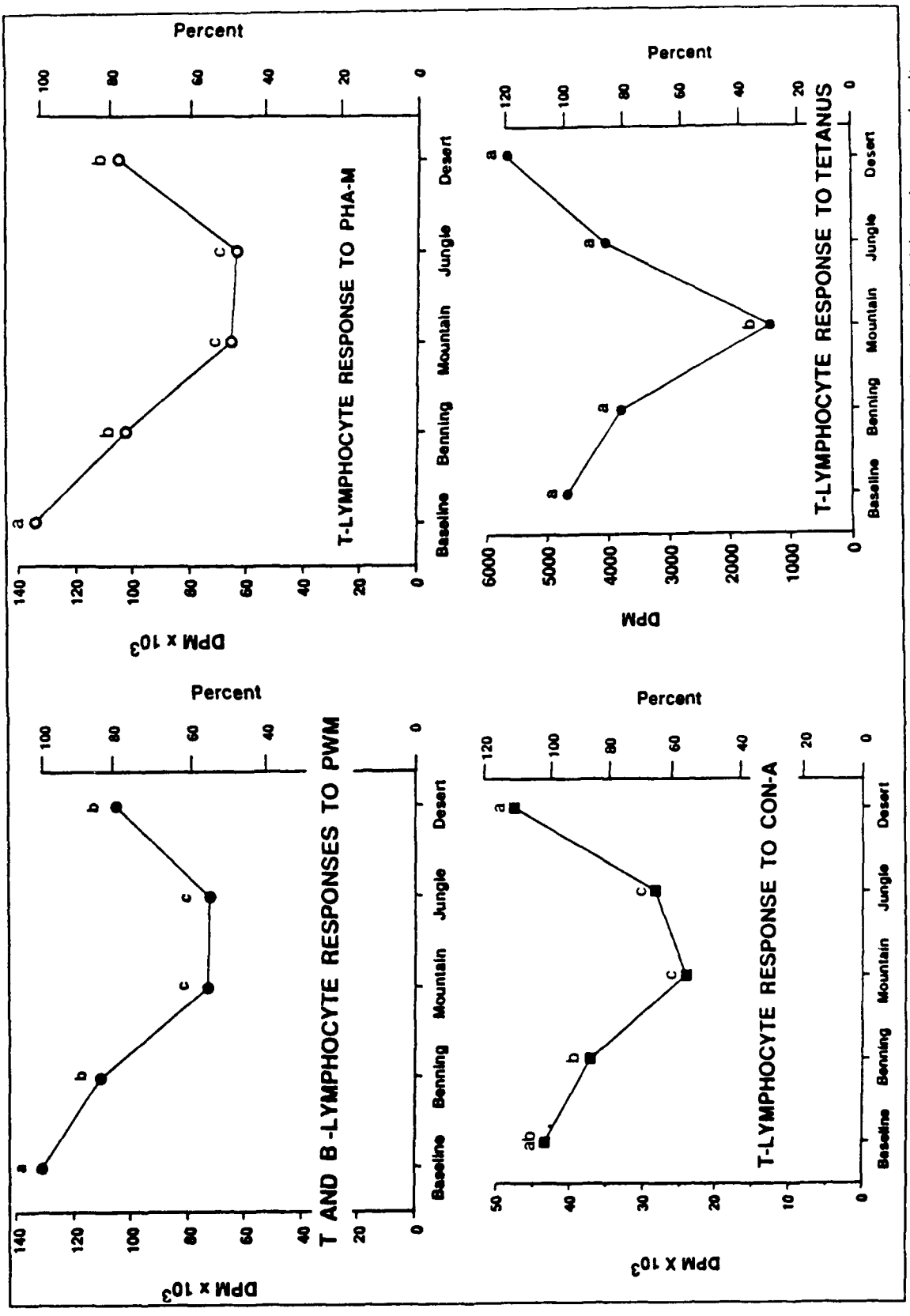

Figure 1. Proliferative activity of lymphocytes collected through the course and stimulated with pokeweed mitogen, phytohemagglutinin, concanavalin A, or tetatus toxoid in vitro. Values with dissimilar letters are different at $p<0.05$.

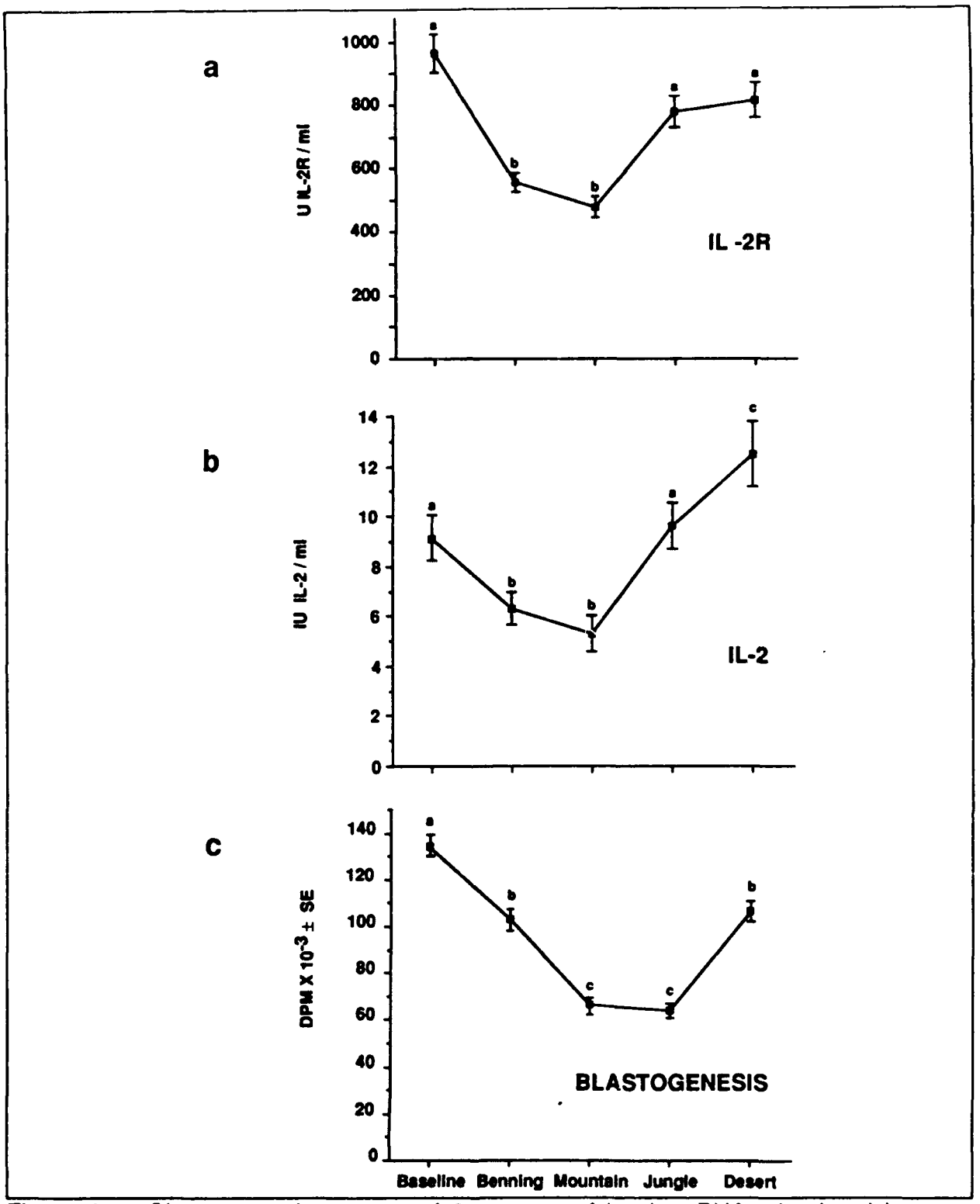

Figure 2. Changes and recovery of 3 aspects of in vitro PHA stimulated immune response: (a) IL-2 receptor secretion, (b) IL-2 secretion, and (c) proliferation.

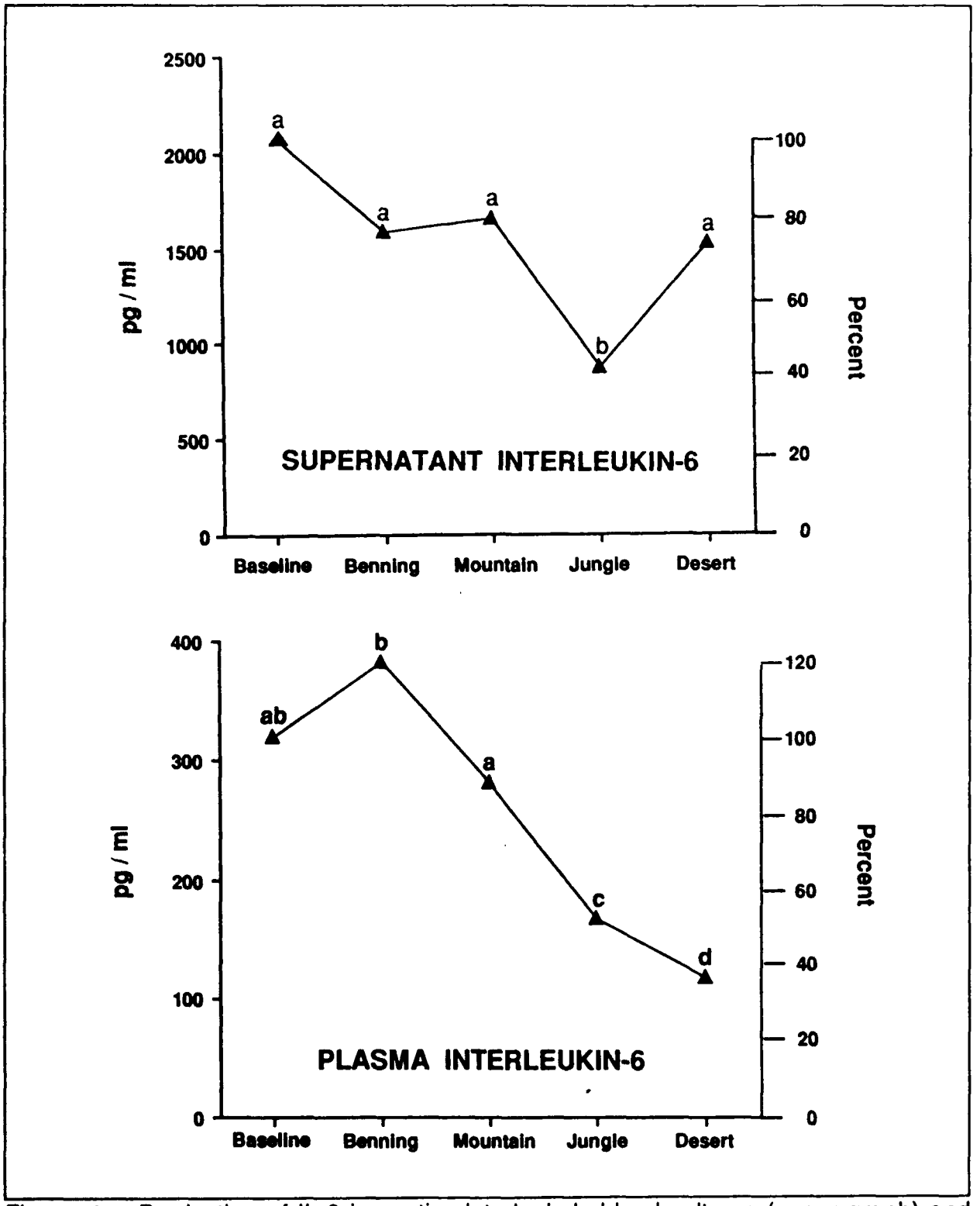

Figure 3. Production of IL-6 in unstimulated whole-blood cultures (upper graph) and plasma levels of IL-6 (lower graph), in samples collected through the course.

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